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Gut microbial species and metabolic pathways associated with response to treatment with immune checkpoint inhibitors in metastatic melanoma

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In patients with metastatic cancer, gut microbiome composition differs between responder and non-responders to immune checkpoint inhibitors. However, there is little consensus on the microbiome taxa associated with response or lack of response. Additionally, recognized confounders of gut microbiome composition have generally not been taken into account. In this study, metagenomic shotgun sequencing was performed on freshly frozen pre-treatment stool samples from 25 patients (12 responders and 13 non-responders) with unresectable metastatic melanoma treated with immune checkpoint inhibitors. We observed no significant differences in alpha-diversity and bacterial prevalence between responders and non-responders ($P > 0.05$). In a zero-inflated multivariate analysis, correcting for important confounders such as age, BMI and use of antibiotics, 68 taxa showed differential abundance between responders and non-responders (false-discovery rate < 0.05). Cox-regression analysis showed longer overall survival for carriers of *Streptococcus parasanguinis* [hazard ratio (HR): 6.9] and longer progression-free survival for carriers of *Bacteroides massiliensis* (HR: 3.79). In contrast, carriership of *Peptostreptococcaceae* (unclassified species) was associated with shorter overall survival (HR 0.18) and progression-free survival (HR 0.11). Finally, 17 microbial pathways differentially abundant between responder and non-responders were observed. These results underline the association between gut microbiome composition and response to immune checkpoint inhibitor therapy in a cohort of patients with cutaneous melanoma. *Melanoma Res* **XXX: 000–000 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Immune checkpoint inhibitors (ICIs) such as anti-PD-1 and anti-CTLA-4 antibodies demonstrate clinical benefit in patients with various solid malignancies, including renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC) and melanoma [1–4]. The efficacy of ICIs has been most strongly established in irresectable cutaneous melanoma, with 1-year overall survival (OS) increasing from ~40% before the era of ICI therapy, to up to 72% now [5]. However, up to 40% of patients with cutaneous melanoma do not respond to ICI therapy and approximately 25% of patients suffer from serious adverse events. A better understanding of the underlying mechanisms that determine the effectiveness of ICIs will help in selecting the right treatment for the right patient and to optimize therapy outcome. Additionally, there is a high grade of so called 'financial toxicity' for ICI therapy [6]. Better selection of patients upfront might helpt to decrease overall costs.

Pre-clinical studies indicated a causal relationship between gut microbiome composition and response to ICI [7–9]. A xenograft model has shown that mice receiving a faecal microbiome transplantation (FMT) from patients responding to ICI therapy showed a lower rate of tumour growth after implantation of melanoma cells compared to mice that received a FMT from non-responding patients [9]. In humans, clinical studies have shown different species, such as *Bifidobacterium adolescentis*, *Holdemania filiformis*, *Faecalibacterium* spp. and taxa belonging to the Ruminococcaceae family, to be more abundant in patients with metastatic melanoma responding to ICIs

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compared to non-responding patients [9–11]. In patients with NSCLC or RCC, higher abundance of species including *Veillonella parvula* and *Akkermansia muciniphila* in the gut was observed in responders to anti-PD-1 compared to non-responders and patients with prolonged survival [12,13]. Taxa more abundant in non-responding patients include taxa belonging to *Actinomycetales* and *Lactobacillaceae* [11].

A complementary method of interrogating the gut microbiome is by evaluation of the functional pathways predicted from microbial genomes [14]. Abundance of functional pathways was shown to be relatively stable among healthy individuals, despite inter-individual variation of the gut taxonomical composition [15]. Therefore, interrogation of these microbial pathways gives additional insight in the functionality of the gut microbiome. Although most studies report taxonomical changes alone, one study has reported that patients with metastatic melanoma that respond to ICI therapy have higher abundance of anabolic microbial pathways compared to non-responders [9].

Due to the lack of a gold-standard, different sample collection methods have been applied. Furthermore, factors that were shown to significantly affect the gut microbiome composition, such as sex, age, BMI, antibiotic (AB) use and proton pump inhibitor (PPI) use are generally not considered [16,17]. In addition, the use of different sequencing technologies impacts the resolution in which a microbial community can be studied [18]. These factors, in addition to differences in statistical methods and small samples sizes hamper direct comparison of study results.

Here, we used a strictly protocolized collection procedure to obtain high-quality fresh frozen stool samples from patients with metastatic melanoma before start of ICI therapy. We collected data on patient factors that influence response to ICIs as well as factors that are known to influence gut microbiome composition. We performed metagenomic shotgun sequencing (MSS) to obtain a high-resolution profile of the gut microbiome. We then assessed the difference in microbial diversity, prevalence and abundance of taxa and of microbial pathways between responders and non-responders to ICI therapy. Finally, we performed an exploratory analysis using a zero-inflated, multivariate model to identify taxa potentially associated with response or non-response, and assessed the association of carriership of significantly associated taxa with progression-free survival (PFS) and OS.

Patients and methods

Patient selection

Patients over the age of 18 with a pathologically confirmed diagnosis of irresectable stage IIIc or stage IV cutaneous melanoma, eligible for systemic ICI therapy (anti-PD-1, anti-CTLA-4 or combination) were approached for study participation. All patients signed informed consent. This study was approved by the UMCG Medical Ethics Committee (registration number 2012/085) and reported to ClinicalTrials.gov (Identifier: NCT02600143).

Clinical and laboratory evaluation

Baseline characteristics such as sex, age, BMI, tumour M-stage and previous anti-cancer therapies were collected. Routine blood biochemistry, including serum lactate dehydrogenase (LDH)-levels, was performed at baseline. Evaluation of clinical symptoms and routine blood biochemistry was performed at the outpatient clinic every 2 or 3 weeks during treatment with the ICI. A list of current medical prescriptions was recorded before the start of therapy and updated during subsequent visits.

Response evaluation

Baseline radiological evaluation, consisting of a computed tomography (CT) scan of the thorax, abdomen and pelvis and MRI of the brain, was performed before start of therapy. Follow-up radiological evaluation was performed every 10–14 weeks as long as the patient received systemic therapy. Additional CT- or MRI scans were performed in case of suspicion of progression. If first radiological evaluation after start of therapy was inconclusive, a confirmatory scan was performed 4 weeks later.

Response was assessed according to response evaluation criteria in solid tumours (RECIST) v1.1. Patients with a confirmed response, defined as a complete response, partial response (PR) or stable disease (SD) according to RECIST 12 weeks after start of therapy that was ongoing at the next radiological evaluation, were labelled 'responder'. In order to include late responders in our analysis, patients with progressive disease (PD) on the first radiological evaluation but a response at the second radiological evaluation compared to baseline were also labelled 'responder'. Patients with PD on the first radiological evaluation that was confirmed on the next follow-up scan, or patients with PD on the first radiological evaluation that were unable to complete a confirmation scan due to clinical progression were labelled 'non-responder'.

Stool sample collection and DNA extraction

Patients received oral and written instructions regarding the stool collection procedure. Patients were instructed to collect 1–2 ml of faeces using a collection kit that could be used at home and to store the sample in the home freezer directly after collection. Patients transported samples to the hospital in a frozen, insulated cooling bag to prevent thawing. After arrival in the hospital the samples were directly stored at –80°C until DNA extraction. Microbial DNA was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) as previously described [19].

Sequencing and microbiome profiling

MSS was performed for all samples using the Illumina HISeq platform at the Broad Institute, Cambridge, MA. Raw data was processed consistently with data analysis of 1000IBD and Lifelines-DEEP cohorts as previously described [16,20,21]. In brief, KneadData tools (v0.5.1) were used to process paired-end metagenomic reads in fastq format by trimming reads to PHRED quality 30, and to remove Illumina adapters [22]. Reads that aligned to human genome were removed from metagenomes (GRCh37/hg19) using KneadData integrated Bowtie2 (v2.3.4.1), and quality of processed samples was examined using FastQC toolkit (v0.11.7) to confirm that sequencing and quality control were successful (all samples were found to confer to 'good quality' FastQC profile) [23,24]. Taxonomical profiling of metagenomes was performed using MetaPhlAn2 tool (v2.7.2), and relative abundances of genes encoding microbial biochemical pathways were calculated using HUMAnN2 pipeline (v0.11.1) integrated with DIAMOND alignment tool (v0.8.22), uniref90 protein database (v0.1.1) and ChocoPhlAn pangenome database (v0.1.1) [25–28]. Analyses were performed using locally installed tools and databases on CentOS (release 6.9).

Assessment of overall gut microbiome composition and differences in prevalence between responders and non-responders

To assess the alpha-diversity, the Shannon-index was calculated for each faecal sample. The difference in Shannon index between responders and non-responders was assessed using the Kruskal–Wallis rank-sum test. To evaluate the difference in prevalence of taxa between responders and non-responders, abundance data was converted to either one (i.e. abundance > 0, taxa present) or 0 (i.e. abundance = 0, taxa not present). Next, we performed a Fisher's exact test and logistic regression analysis to assess the difference in prevalence between responders and non-responders for each taxa individually. Correction for multiple testing was performed using the Holm method. Statistical cut-off for nominal and corrected P values was set at $P < 0.05$.

Assessment of relative microbial abundance between responders and non-responders

First, we performed an univariate linear regression analysis to assess the difference in relative abundance of taxa between responders and non-responders. Next, to correct for important confounding factors, we performed a multivariate linear regression analysis with covariates age, sex, BMI, M-stage (AJCC version 8), LDH-level (>250 U/L vs. <250 U/L), previous anti-melanoma therapy, systemic therapy (anti-PD1 or anti-PD-1/anti-CTLA-4 combination), AB use (yes/no), PPI use (yes/no) and colitis during ICI therapy (yes/no, any grade). In order to account for the high number of zero-valued observations in the dataset, we utilized a zero-inflated model. All linear models were created utilizing the 'Multivariate Association with Linear Models' (MaAsLin package for *R*) [28]. False-discovery rate (FDR) corrected P values were calculated using the Benjamini–Hochberg method. Statistical cut-off was set at FDR <0.05.

Association with overall and progression-free survival

To evaluate the possibility of an association between taxa carriership and OS or PFS, we performed an univariate Cox-Regression analysis for all taxa differentially abundant between responders and non-responders (FDR < 0.05). Next, we performed a multivariate Cox regression analysis for those taxa significantly associated in the univariate analysis, including relevant variables (those associated with OS or PFS according to univariate Cox regression analysis) as covariates. Statistical cut-off for P values was set at 0.05. For taxa associated with OS or PFS, Kaplan–Meier curves were plotted. All statistical analyses were performed with *R* studio (v.1.0.143).

Results

Baseline patient characteristics and response assessment

Pre-treatment stool samples were obtained from 25 patients (Table 1). We observed no significant differences in baseline characteristics between responders vs. non-responders. Twenty-three patients were treated with anti-PD-1 monotherapy and two with anti-PD-1/anti-CTLA-4 combination therapy.

Radiological evaluation consisting of a CT-scan of the thorax, abdomen and pelvis and MRI of the brain was performed before start of therapy and every 10–14 weeks thereafter for as long as therapy continued. Ten out of 25 patients showed a confirmed response and were labelled 'responder' (Table S1, Supplemental digital content 1, *http://links.lww.com/MR/A205*). In addition, two patients with PD on the first scan showed PR on their confirmatory scan and were also labelled 'responder'. Three patients showed PD on the first scan that was confirmed on a follow-up scan. These patients were labelled 'non-responder'. Seven patients had PD on the first scan and did not complete a follow-up scan due to disease progression. These patients were also labelled 'non-responder'. One patient showed rapid clinical and biochemical progression without evidence for treatment-related toxicity. This patient was unable to complete the first scan and was labelled 'non-responder'. Finally, two patients showed SD on the first scan. One patient showed PD on the confirmatory scan. The other patient was unable to complete the confirmatory scan due to clinical and biochemical deterioration without evidence of toxicity, followed by the patient's death 11 weeks later. Both patients were labelled 'non-responder'. In total, 12 out of 25 patients were identified as 'responder' and 13 patients as 'non-responder'.

Table 1 Baseline characteristics of all patients ($n = 25$)

	Responders ($n = 12$)	Non-responders ($n = 13$)	P value
Sex, *n* (%)			1.0[a]
Male	5 (42)	5 (39)	
Female	7 (58)	8 (62)	
Age (years), median (range)	61.5 (41–72)	64 (21–83)	0.8[b]
BMI, median (range)	26.8 (19.2–34.4)	26.0 (23.9–33.9)	0.7[b]
M-stage at diagnosis[c], n (%)			
M1a	3 (25)	1 (8)	0.6[a]
M1b	3 (25)	5 (39)	
M1c	3 (25)	5 (39)	
M1d	3 (25)	2 (15)	
BRAF mutation status, *n* (%)			
Mutated	8 (67)	9 (69)	1.0[a]
Wildtype	4 (33)	4 (31)	
Medication use, *n* (%)			
Abs	0 (0)	1 (8)	1.0[a]
PPIs	2 (17)	3 (23)	1.0[a]
Corticosteroids	2 (17)	2 (15)	1.0[a]
Serum LDH, *n* (%)			
Normal (≤250 U/L)	11 (92)	10 (77)	0.6[a]
Elevated (>250 U/L)	1 (8)	3 (23)	
Previous anti-melanoma therapy, *n* (%)			
Anti-CTLA-4 therapy	1 (8)	2 (15)	1.0[a]
BRAF-inhibition (± MEK-inhibition)	1 (8)	4 (31)	0.3[a]
Systemic therapy, *n* (%)			
Anti-CTLA-4/anti-PD-1 combination	2 (17)	0 (0)	0.3[a]
Anti-PD-1	10 (83)	13 (100)	

AB, antibiotic; LDH, lactate dehydrogenase; PPI, proton pump inhibitor.
[a]Fisher exact test.
[b]Kruskal–Wallis test.
[c]AJCC eighth edition.

Microbial abundance and microbial pathway abundance in pre-treatment samples

A total of 790 taxa were identified. Taxa present in less than 25% of samples were excluded. Additionally, to include taxa only present in either responders or non-responders, taxa present in at least 50% of responders or non-responders were included, resulting in 192 bacterial taxa included in this analysis (Fig. 1a).

For the microbial pathways, we applied the same selection criteria described above, resulting in 260 microbial pathways available for testing.

Overall gut microbiome composition

At the species level, there was an even distribution of most taxa (Fig. 1c). We observed no taxa exclusively abundant in responders or non-responders at the species level. No significant difference in alpha-diversity (Shannon diversity index) was observed between responders and non-responders (median responders: 2.8, non-responders: 2.7, Kruskal–Wallis $P = 0.46$; Fig. 2).

The predominant phyla in both responders and non-responders were Firmicutes (mean relative abundance of 64% in responders and 61% in non-responders), Actinobacteria (mean relative abundance of 18% in responders and 24% in non-responders) and Bacteroidetes (mean relative abundance of 10% in responders and 10% in non-responders) (Fig. 1b). Taxa belonging to Verrucomicrobia were observed in 12 patients, with a mean relative abundance of 0.3% in responders and 0.5% in non-responders.

Differences in taxa prevalence between responders and non-responders

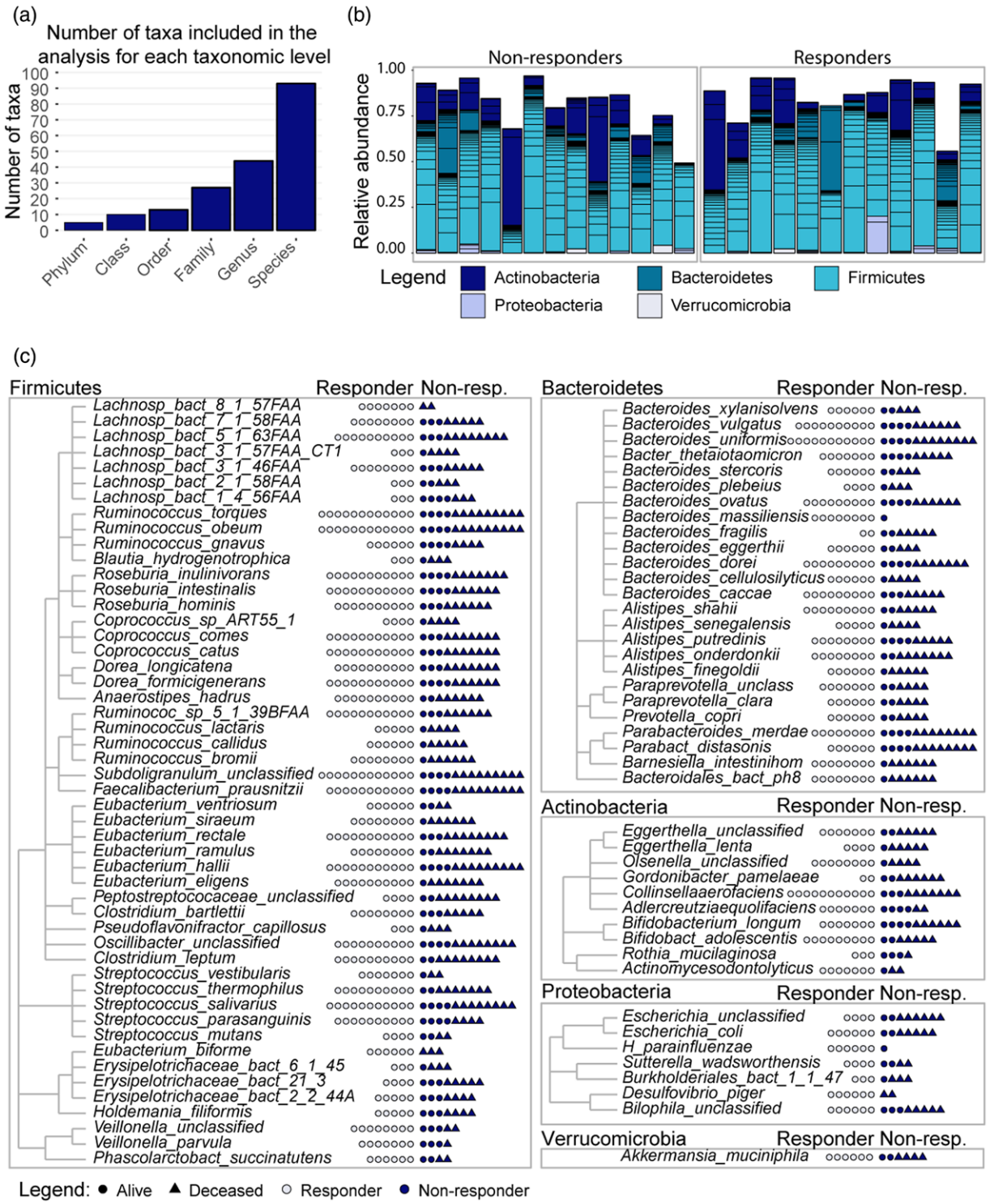
We observed a significant difference (Fisher's exact $P < 0.05$) in the prevalence of *Bacteroides massiliensis*, *Haemophilus parainfluenzae* (association also observed at the genus, family and order level), an unclassified *Peptostreptococcaceae* species, *L. bacterium 8 1 57FAA*, *Parabacteroides distasonis* and *Gordonibacter pamelaeae* (association also observed at the genus level; Table 2). Univariate logistic regression analysis for prevalence of taxa showed a significant association ($P < 0.05$) for *H. parainfluenzae* (association also observed at the genus, family and order level), *Peptostreptococcaceae* (unclassified species), *L. bacterium 8 1 57FAA*, *P. distasonis* and *G. pamelaeae* (association also observed at the genus level; Table 3). Again, significance was lost when correcting for multiple testing.

Results for all taxa are listed in Table S2 (Supplemental digital content 2, *http://links.lww.com/MR/A206*) and Table S3 (Supplemental digital content 3, *http://links.lww.com/MR/A207*).

Differences in taxa abundance between responders and non-responders

When performing an univariate linear regression analysis, we observed a significant association with response for *B. massiliensis* and *Eubacterium biforme* (nominal $P < 0.05$). However, after correction for multiple testing, this association was lost (FDR > 0.05; Table S4, Supplemental digital content 4, *http://links.lww.com/MR/A208*). Since

Fig. 1



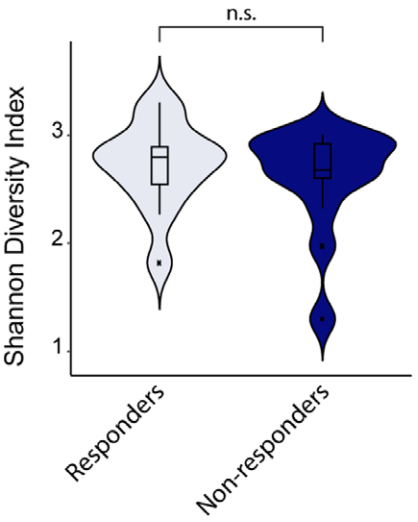
Overview of gut microbiome composition in responders and non-responders. (a) Number of remaining samples after quality control and filtering on bacterial taxa. Taxa available in less than 25% of all samples were excluded. Additionally, taxa available in at least 50% of all responders or 50% of all non-responders were added, to avoid removal of taxa mutually exclusive in either responders or non-responders. A total of 192 taxa remained available for testing. (b) Overview of the relative abundance (y-axis) for taxa at the species level for each sample (x-axis). The colours depict the phyla to which the species belong. (c) Taxonomic tree of taxa available for testing, divided by phylum (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia). The samples that included the taxa are shown as a light blue (responder) or dark blue (non-responder) circle (patient alive at last follow-up) or triangle (patient deceased at last follow-up). Samples that did not include the taxa are not shown.

microbial profiles usually contain a large amount of zero-valued observations, we then applied a zero-inflated model to the abundance data.

To correct for variables that are likely to influence gut microbiome composition or response to ICIs, we performed a multivariate linear regression analysis with covariates age, sex, drug use (PPIs, ABs), BMI, melanoma M-stage, systemic therapy, pre-treatment serum LDH-level, colitis during treatment and previous anti-melanoma therapy (BRAF- or CTLA-4-inhibition). When corrected for multiple testing, we observed a significant correlation (FDR < 0.05) for the relative abundance of 68 unique taxa with response to ICI therapy (Tables 4 and 5). Of these, 27 taxa showed a positive correlation with response and the remaining 41 taxa showed a negative correlation with response. Mean relative abundance of 40 taxa (27 species, nine genera, three families and one class) was increased in responders, and mean relative abundance of 28 taxa was increased in non-responders (19 species, four genera, four families and one order) (Fig. 3). At the species level, we found the highest mean relative abundance for *Ruminococcus gnavus*, *Escherichia coli*, *E. biforme*, *Phascolarctobacterium succinatutens* and *Streptococcus salivarius* in responders and *Bifidobacterium longum*, *Prevotella copri*, *Coprococcus sp ART55-1*, *Eggerthella unclassified* and *Eubacterium ramulus* in non-responders (Fig. 3).

Results for all significantly associated taxa are listed in Table S5 (Supplemental digital content 5, *http://links.lww.com/MR/A209*).

Fig. 2



Shannon index (y-axis) for microbial diversity in responders (light blue) and non-responders (dark blue). We observed no significant difference between groups (Kruskal–Wallis $P = 0.46$).

Association of microbial taxa with progression-free survival and overall survival

We observed a longer PFS for carriers of *B. massiliensis* compared to non-carriers [Univariate Cox-Regression Wald-test $P = 0.04$, HR: 3.79, 95% confidence interval (CI): 1.06–13.52] (Fig. 4a and Table S6, Supplemental digital content 6, *http://links.lww.com/MR/A210*). In contrast, carriers of *Peptostreptococcaceae* (unclassified species) showed a significantly shorter PFS compared to non-carriers [Univariate Cox-Regression Wald-test $P = 0.007$, hazard ratio (HR): 0.18, 95%CI: 0.05–0.62] (Fig. 4b). All other taxa showed no significant association with PFS. The univariate analysis showed no significant association with PFS for any of the included variables (i.e. sex, age, tumour M-stage, current systemic therapies, previous treatment with BRAF- or CTLA-4-inhibition, corticosteroid use, LDH-level and BMI), therefore no multivariate Cox regression analysis was performed.

Table 2 Results from the Fisher's exact test for the association between presence of taxa and response to immune checkpoint inhibitor therapy

Level	Taxa		Responders	Non-responders	Nominal *P* value[a]	Corrected *P* value
Order	Pasteurellales	Carrier	6	1	0.03	1
		Non-carrier	6	12		
Family	Pasteurellaceae	Carrier	6	1	0.03	1
		Non-carrier	6	12		
Genus	*Haemophilus*	Carrier	6	1	0.03	1
		Non-carrier	6	12		
	Gordonibacter	Carrier	2	8	0.041	1
		Non-carrier	10	5		
Species	*Bacteroides massiliensis*	Carrier	8	1	0.004	0.62
		Non-carrier	4	12		
	Haemophilus parainfluenzae	Carrier	6	1	0.03	1
		Non-carrier	6	12		
	Parabacteroides distasonis	Carrier	6	12	0.03	1
		Non-carrier	6	1		
	Gordonibacter pamelaeae	Carrier	2	8	0.041	1
		Non-carrier	10	5		
	Lachnospiraceae bacterium 8 1 57FAA	Carrier	7	2	0.041	1
		Non-carrier	5	11		
	Peptostreptococcaceae unclassified	Carrier	4	10	0.047	1
		Non-carrier	8	3		

[a]Fisher's exact test.

Table 3 Results from the logistic regression analysis for the association between presence of taxa and response to immune checkpoint inhibitor therapy

Level	Taxa	Coefficient[a]	Nominal *P* value	Corrected *P* value
Order	Pasteurellales	2.48	0.037	0.7
Family	Pasteurellaceae	2.48	0.037	0.7
Genus	*Gordonibacter*	−2.08	0.031	0.7
	Haemophilus	2.48	0.037	0.7
Species	*Gordonibacter pamelaeae*	−2.08	0.031	0.7
	Peptostreptococcaceae noname unclassified	−1.90	0.035	0.7
	Lachnospiraceae bacterium 8 1 57FAA	2.60	0.036	0.7
	Parabacteroides distasonis	−2.48	0.037	0.7
	Haemophilus parainfluenzae	2.48	0.037	0.7

[a]Reference group: responders.

Table 4 Overview of taxa with a significant positive correlation (i.e. response to immune checkpoint inhibitor therapy is associated with higher relative abundance) between relative abundance and response to immune checkpoint inhibitor therapy in a multivariate linear model

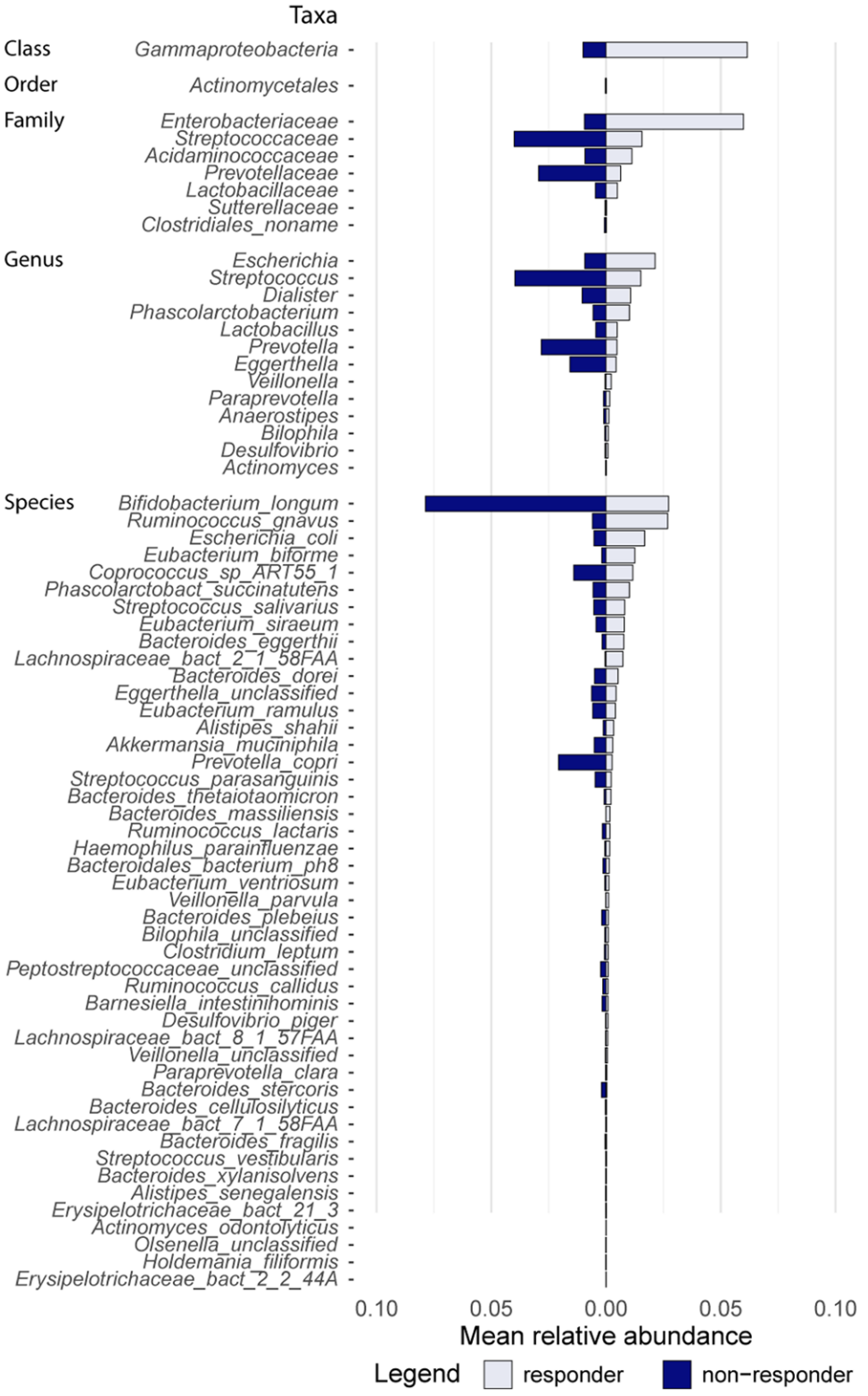
Taxonomic level	Taxa	Coefficient[a]	FDR
Genus	*Phascolarctobacterium*	0.31	<0.001
	Dialister	0.56	<0.001
	Anaerostipes	0.95	0.032
	Veillonella	3.77	<0.001
Species	*Lachnospiraceae bacterium 2 1 58FAA*	0.08	<0.001
	Eggerthella unclassified	0.09	<0.001
	Bacteroides eggerthii	0.11	<0.001
	Lachnospiraceae bacterium 8 1 57FAA	0.19	<0.001
	Bacteroides massiliensis	0.21	<0.001
	Phascolarctobacterium succinatutens	0.31	<0.001
	Eubacterium siraeum	0.42	0.002
	Olsenella unclassified	0.46	<0.001
	Bacteroides thetaiotaomicron	0.74	<0.001
	Coprococcus sp ART55 1	0.90	<0.001
	Eubacterium biforme	1.10	<0.001
	Bacteroides dorei	1.21	0.009
	Ruminococcus lactaris	1.22	<0.001
	Peptostreptococcaceae noname unclassified	1.30	<0.001
	Veillonella parvula	1.45	<0.001
	Eubacterium ventriosum	1.49	<0.001
	Lachnospiraceae bacterium 7 1 58FAA	2.14	0.002
	Erysipelotrichaceae bacterium 21 3	2.15	<0.001
	Akkermansia muciniphila	2.68	<0.001
	Veillonella unclassified	3.60	<0.001
	Erysipelotrichaceae bacterium 2 2 44A	5.14	<0.001
	Ruminococcus gnavus	10.28	<0.001
	Desulfovibrio piger	14.67	<0.001

FDR, false-discovery rate.
[a]Reference group: non-responders.

A significantly longer OS was observed for carriers of *Streptococcus parasanguinis* compared to non-carriers (Wald-test $P = 0.017$, HR: 5.05, 95%CI: 1.33–19.21). In line with abovementioned association, carriers of *Peptostreptococcaceae* (unclassified species) showed a significantly shorter OS compared to non-carriers (Wald-test $P = 0.046$, HR: 0.12, 95%CI: 0.01–0.96). Additionally, a baseline serum LDH level of > 250U/L was associated with OS (Wald-test $P = 0.036$) in an univariate Cox-regression analysis. None of the other taxa or other variables tested was associated with OS. When performing a multivariate analysis for each significantly associated taxon independently, with covariate baseline serum LDH level, both species remained significantly associated with OS (*S. parasanguinis* Wald-test $P = 0.008$, HR: 6.9, 95%CI: 1.63–29.14, *Peptostreptococcaceae* (unclassified species) Wald-test $P = 0.018$, HR: 0.11, 95%CI: 0.01–0.93; Fig. 4c and d). Of note, we observed a significant association between OS and abundance of the genus *Anaerostipes* in the univariate analysis, but this association was lost when correcting for baseline serum LDH levels (Table S6, Supplemental digital content 6, *http://links.lww.com/MR/A210*).

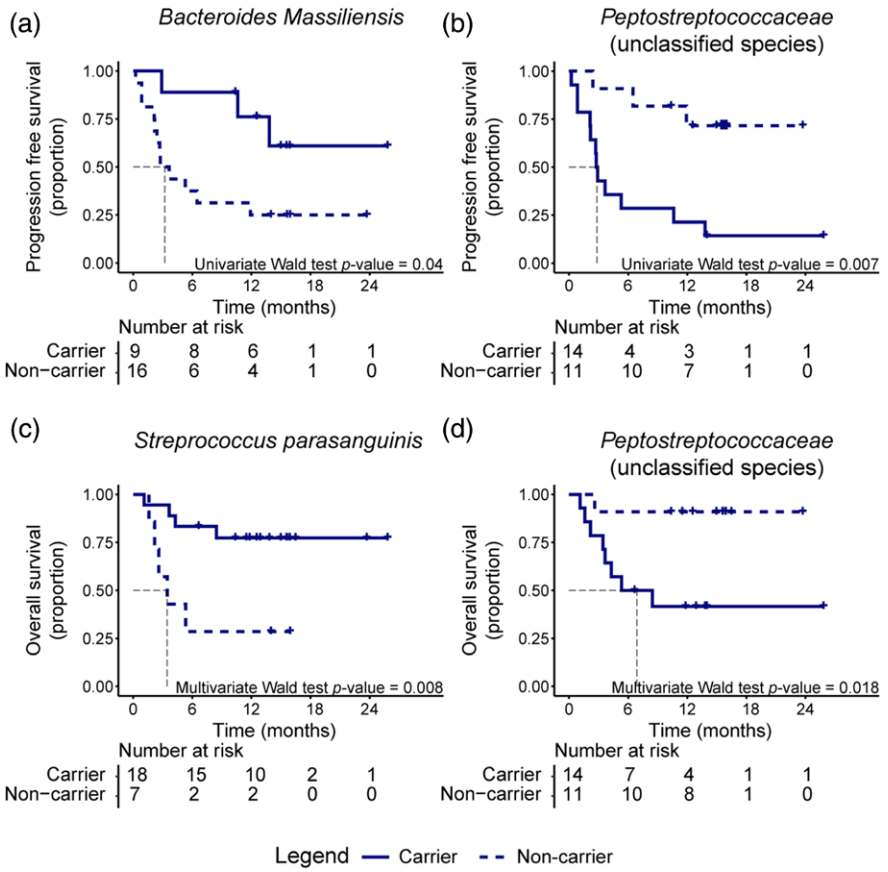
Interestingly, when performing a separate multivariate Cox regression analysis for each taxon that showed differential abundance in responders or non-responders (FDR < 0.05) with baseline serum LDH level entered as covariate, we observed a difference in OS between carriers and non-carriers of an additional five taxa, including *V. parvula* (Wald-test $P = 0.016$, HR: 8.51, 95%-CI: 1.04 – 69.61), *Actinomyces odontolyticus* (Wald-test $P = 0.02$, HR: 8.5, 95%CI: 1.18–61.6) (NB. Association also found at the genus and family level) and *Lactobacillaceae* (Wald-test *P*

Fig. 3



Taxa significantly more abundant in either responders or non-responders (FDR cut-off = 0.05). The x-axis depicts the mean relative abundance in responders (light blue) and non-responders (dark blue). Species are listed on the y-axis. FDR, false-discovery rate.

Fig. 4



Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) of taxa differentially abundant in responders vs. non-responders and significantly associated with progression free or OS in a Cox-regression analysis (Wald test $P < 0.05$). (a) Longer PFS was observed for carriers of *Bacteroides massiliensis* compared to non-carriers. (b) Conversely, compared to carriers of *Peptostreptococcaceae* (unclassified species) longer PFS for non-carriers was observed. (c) Carriers of *Streptococcus parasanguinis* showed significantly longer OS compared to non-carriers. (d) Similarly to the association with PFS, non-carriers of *Peptostreptococcaceae* (unclassified species) showed longer OS compared to carriers.

= 0.02, HR: 4.12, 95%CI: 1.03 – 16.42). None of these taxa showed associations with OS in the univariate Cox regression analysis (Table S6, Supplemental digital content 6, *http://links.lww.com/MR/A210*).

Overall composition of microbial pathway abundances

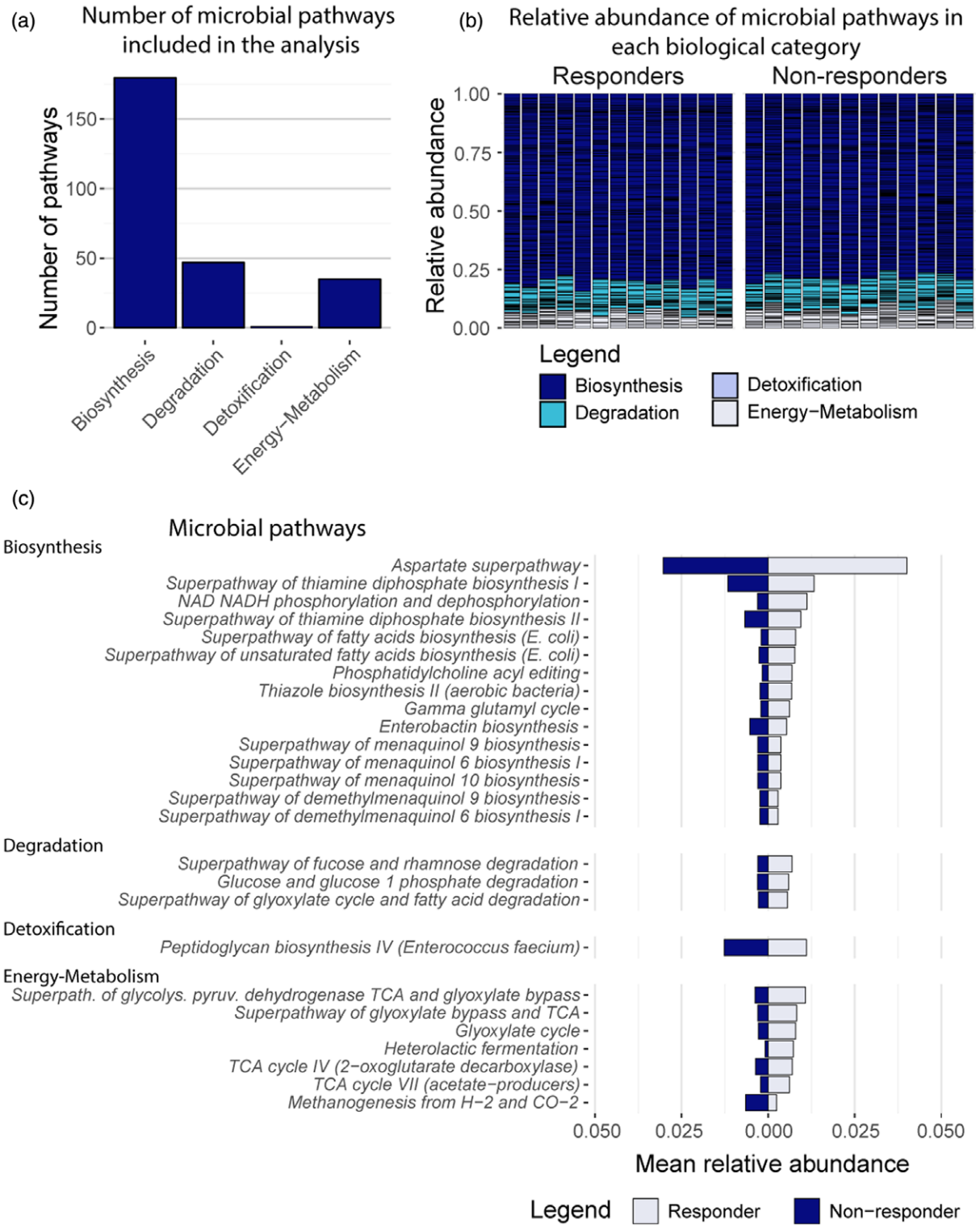
Of all pathways included, 177 were related to biosynthesis, 47 to degradation, one to detoxification and 35 to energy-metabolism (Fig. 5a). Pathways related to biosynthesis were most predominant in all samples, both in responders and non-responders (79%; Fig. 5b). Pathways related to degradation and energy-metabolism made up 13 and 8% of the samples, respectively. Finally, pathways related to detoxification showed limited relative abundance of 0.1%.

Differences in abundance of microbial pathways between responders and non-responders

We observed no significant differences in microbial pathway abundance between responders and non-responders using univariate and multivariate linear models (Table S7, Supplemental digital content 7, *http://links.lww.com/MR/A211*).

Utilizing a zero-inflated model, as described above, and maintaining an FDR of 0.05, we observed 17 pathways with a higher mean relative abundance in responders compared to non-responders and two pathways with a higher mean relative abundance in non-responders compared to responders (Fig. 5c). The five highest mean relative abundances in responders were observed for aspartate superpathway (PWY0_781), superpathway of thiamine diphosphate biosynthesis I (THISYN_PWY), NAD/NADH phosphorylation and dephosphorylation (PWY_5083), superpathway of glycolysis, pyruvate dehydrogenase, TCA and glyoxylate bypass (GLYCOLYSIS_TCA_GLYOX_BYPASS) and superpathway of thiamine diphosphate biosynthesis II (PWY_6895). For non-responders, only two pathways showed higher mean relative abundance compared

Fig. 5



Overview of abundance of microbial pathways in responders vs. non-responders. (a) Number of microbial pathways per biologic category available for testing. (b) Overview of the relative abundance (y-axis) for microbial pathways for each sample (x-axis). The colours depict the biological categories to which the pathways belong. (c) Mean abundance of microbial pathways with significant (FDR < 0.05) higher abundance in responders (light blue) or non-responders (dark blue). The x-axis depicts the mean abundance; the y-axis lists the microbial pathways, divided by biologic category (i.e. biosynthesis, degradation, detoxification and energy-metabolism). FDR, false-discovery rate.

to responders, namely peptidoglycan biosynthesis IV (PWY_6471) and methanogenesis from H_2 and CO_2 (METHANOGENESIS_PWY).

Results for all pathways are listed in Table S8 (Supplemental digital content 8, *http://links.lww.com/MR/A212*).

Discussion

In this study, we observed an association between the relative abundance of 68 individual taxa in the gut microbiome and response to ICI therapy. In addition, we observed prolonged OS for carriers of *S. parasanguinis*, prolonged PFS for carriers of *B. massiliensis* and shorter OS and PFS for carriers of *Peptostreptococcaceae* (unclassified species) compared to non-carriers. Finally, we show differential abundance of 17 microbial pathways in the gut of responders compared to non-responders.

In accordance with previous studies, we found the relative abundance of *V. parvula* and *Bacteroides thetaiotaomicron* to be positively correlated with response to ICI therapy (coefficient > 0; Table 4). Additionally, as observed in previous studies, relative abundance of *E. coli* and *A. odontolyticus* was negatively correlated with response to ICI therapy (coefficient < 0; Table 5) [9,11–13]. Furthermore, we observed a positive correlation between high relative abundance of *A. muciniphila* and response to ICI therapy. This association has previously only been described in patients with NSCLC or RCC. In contrast to previous findings, we observed high abundance of *Bacteroides eggerthii* to be correlated with response, while in previous studies *B. eggerthii* has been associated with non-response [11]. Conversely, high relative abundance of *S. parasanguinis*, *H. filiformis* and *B. longum* taxa that have previously been associated with response, showed a negative correlation with response to ICI therapy in the present study [11,13]. In part, this may be due to differences in study population (e.g. differences in cancer type and systemic therapy). Additionally, variations in stool sample collection procedures, definitions of response and statistical analyses are also likely to have an effect.

Finally, correction for confounding factors that influence either gut microbiome composition or response to ICIs is not widely implemented. For instance, when correcting for confounding factors such as use of ABs, BMI and age, we observed a shift in direction of the correlation (i.e. from positive to negative) at the genus level for *Phascolarctobacterium* (univariate analysis results: coefficient: –0.91; multivariate analysis results: coefficient: 0.31). This underlines the importance of taking confounders into account when interrogating gut microbiome composition.

In addition to the correlation between taxa abundance and response to ICI therapy, we were able to show an association with OS or PFS for three individual taxa. It should be noted that although higher relative abundance of *Peptostreptococcaceae* (unclassified species) was positively correlated with response to ICI therapy, we observed shorter PFS for carriers vs. non-carriers. Similarly, for *S. parasanguinis* a negative correlation with response to ICI therapy was observed, yet longer OS for carriers vs. non-carriers was observed. These differences could be attributable to the difference in prevalence (carrier vs non-carrier) and relative abundance of these taxa.

Currently, FMT studies in patients with metastatic melanoma are recruiting (ClinicalTrials.gov Identifiers: NCT03353402 and NCT03341143). However, despite the rapid developments in this field of research, there is still little overlap in taxa that are found to be associated with response or non-response to ICI therapy between studies (Figure S1, Supplemental digital content 9, *http://links.lww.com/MR/A213*) [9–13]. Therefore, there remains a need for larger studies with standardized collection

Table 5 Overview of taxa with a significant negative correlation (i.e. non-response to immune checkpoint inhibitor therapy is associated with higher relative abundance) between relative abundance and response to immune checkpoint inhibitor therapy in a multivariate linear model

Taxonomic level	Taxa	Coefficient[a]	FDR
Class	Gammaproteobacteria	−0.72	0.001
Order	Actinomycetales	−0.41	<0.001
Family	Prevotellaceae	−1.68	<0.001
	Streptococcaceae	−1.14	0.001
	Clostridiales noname	−0.75	<0.001
	Enterobacteriaceae	−0.67	<0.001
	Acidaminococcaceae	−0.54	<0.001
	Lactobacillaceae	−0.35	0.031
	Sutterellaceae	−0.06	<0.001
Genus	*Desulfovibrio*	−13.32	<0.001
	Eggerthella	−3.06	0.043
	Prevotella	−1.79	<0.001
	Streptococcus	−1.16	0.001
	Actinomyces	−0.74	<0.001
	Escherichia	−0.67	0.001
	Paraprevotella	−0.39	0.038
	Lactobacillus	−0.37	0.026
	Bilophila	−0.36	<0.001
Species	*Ruminococcus callidus*	−35.23	<0.001
	Escherichia coli	−5.10	<0.001
	Haemophilus parainfluenzae	−3.36	<0.001
	Streptococcus parasanguinis	−2.42	<0.001
	Bifidobacterium longum	−2.27	0.002
	Barnesiella intestinihominis	−2.02	<0.001
	Bacteroides plebeius	−1.43	<0.001
	Bilophila unclassified	−1.39	<0.001
	Streptococcus salivarius	−1.06	0.001
	Bacteroidales bacterium ph8	−1.03	<0.001
	Alistipes shahii	−0.96	0.007
	Eubacterium ramulus	−0.95	0.001
	Alistipes senegalensis	−0.93	<0.001
	Bacteroides stercoris	−0.83	<0.001
	Paraprevotella clara	−0.73	<0.001
	Bacteroides xylanisolvens	−0.62	<0.001
	Clostridium leptum	−0.60	0.029
	Holdemania filiformis	−0.55	<0.001
	Prevotella copri	−0.54	<0.001
	Actinomyces odontolyticus	−0.40	<0.001
	Bacteroides cellulosilyticus	−0.06	<0.001
	Streptococcus vestibularis	−0.05	<0.001
	Bacteroides fragilis	−0.03	<0.001

FDR, false-discovery rate.
[a]Reference group: non-responders.

procedures, well defined response definitions and uniform statistical methodology. Finally, standard prospective collection of data on important confounding factors such as diet, drug use and BMI is of great importance in gut microbiome studies. The collection of data in this study will continue until 300 patients have been included.

In conclusion, we observed 68 unique taxa to be associated with response to ICI therapy in patients with unresectable metastastic melanoma, including the previously described association of *V. parvula*, *B. thetaiotaomicron*, *A. muciniphila*, *E. coli* and *A. odontolyticus*. In addition, we observed prolonged OS for carriers of *S. parasanguinis*, prolonged PFS for carriers of *B. massiliensis* and shorter OS and PFS for carriers of *Peptostreptococcaceae* (unclassified species) compared to non-carriers. Finally, we observed an association between abundance of 17 microbial pathways and response to ICI therapy. These results underline the association between gut microbiome composition and response to ICI therapy in a cohort of patients with cutaneous melanoma. There remains a need for large, prospective studies that take into account important confounding factors in order to confirm these and previously published results.

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Conflicts of interest

G.A.P.H. received research funding from BMS (payment to the institution) and is an advisory board member for Bristol-Myers Squibb and Merck Sharp & Dohme. M.J. is an advisory board member for Bristol-Myers Squibb and Merck Sharp & Dohme. R.K.W. received consultancy fees from Takeda Pharmaceuticals and unrestricted research grants from Takeda Pharmaceuticals, Johnson and Johnson, Tramedico and Ferring. For the remaining authors, there are no conflicts of interests.

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